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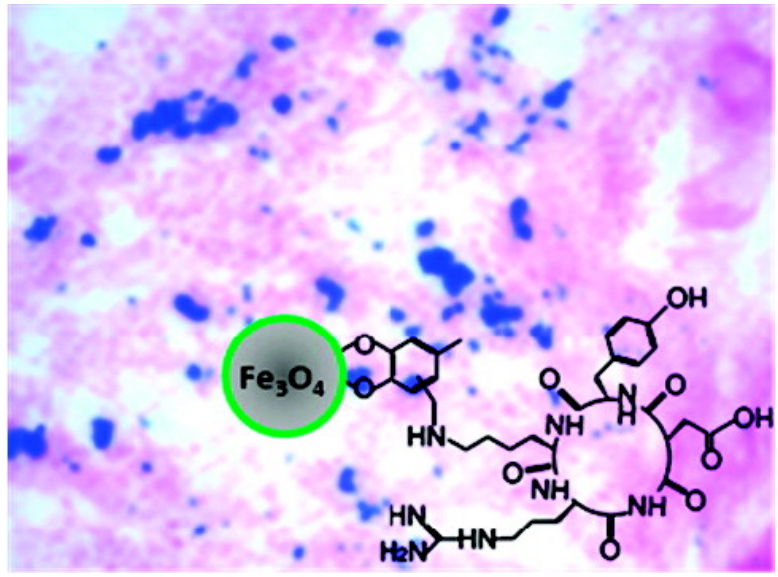

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Ultrasmall c(RGDyK)-Coated Fe_3O_4 Nanoparticles and Their Specific Targeting to Integrin $\alpha_v\beta_3$-Rich Tumor Cells

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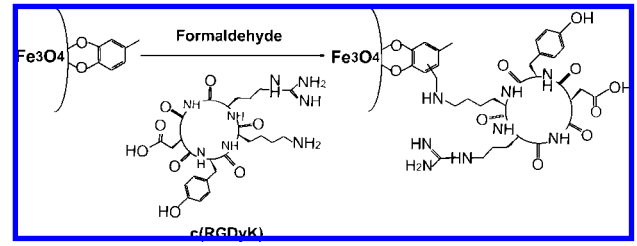
Noninvasive detection of tumors at an early stage by magnetic resonance imaging (MRI) needs highly sensitive contrast agents. Synthesized from solution phase and coated with various hydrophilic molecules, iron oxide nanoparticles (IONPs) can be well stabilized in biological systems and used for T_2 contrast enhancement in MRI.[1−3] However, commonly used IONP contrast agents require nonspecific uptake by mononuclear phagocytes to improve the local contrast, and at the hydrodynamic size of over 50 nm, these particles have very limited extravasation ability and are subject to easy uptake by the reticuloendothelial system (RES),[4,5] which undermines severely their targeting specificity. To overcome the problems of nonspecific uptake and to enhance the extra-vascular ability, even smaller hydrodynamic sizes are desired.[5,6] Despite recent synthetic progresses, making small (<10 nm hydrodynamic size) IONPs with required biocompatibility and targeting capability is still extremely challenging.

Here, we present a direct synthesis of ultrasmall Fe_3O_4 NPs (<10 nm in hydrodynamic diameter) and demonstrate their in vivo tumor-specific targeting capability. The Fe_3O_4 NPs with 4.5 nm core size were synthesized by thermal decomposition of iron pentacarbonyl, $Fe(CO)_5$, followed by air oxidation. Different from any of the previous synthesis methods,[7] the current preparation used a new ligand 4-methylcatechol (4-MC) as the surfactant. More importantly, the 4-MC coated IONP surface was directly conjugated with a peptide, c(RGDyK), via the Mannich reaction, rendering the particles stable in physiological environment. The synthesis is illustrated in Scheme 1. The c(RGDyK)-MC-Fe_3O_4 NPs have an overall diameter of ∼8.4 nm and are stable in physiological conditions. They can target specifically to integrin $\alpha_v\beta_3$-rich tumor cells. When administrated intravenously, these c(RGDyK)-MC-Fe_3O_4 NPs accumulate preferentially in tumor cells, which are readily tracked by MRI.

Fe_3O_4 NPs were synthesized by thermal decomposition of $Fe(CO)_5$ in benzyl ether in the presence of 4-MC followed by air oxidation.[8] Because of the formation of a strong chelate bond between iron and a catechol unit,[9] 4-MC forms a tight thin coating layer around the NP surface. The sizes of the NPs (from 2.5−5 nm as shown in Figure S1) were tunable by the MC/Fe ratio. Figure 1A shows the high resolution transmission electron microscopy (HRTEM) image of the 4.5 nm Fe_3O_4 NPs. The inverse spinel structure of the Fe_3O_4 NPs was characterized by X-ray diffraction (XRD) analysis (Figure S2). The 4-MC coating was confirmed by FTIR (Figure 1B), from which three aromatic C=C stretch peaks at 1602, 1521, and 1415 cm^{-1} are attributed to the coated 4-MC and are right-shifted compared to those from the free 4-MC (1623, 1527, and 1448 cm^{-1}). Peaks at 624, 594, and 445 cm^{-1} are typical Fe−O absorption bands.[10] The MC-Fe_3O_4 NPs were well dispersed in chloroform ($CHCl_3$) or dimethylformamide (DMF), ready for further functionalization.

Scheme 1. Schematic Illustration of Coupling c(RGDyK) Peptide to Fe_3O_4 NPs



The aromatic ring of 4-MC around the Fe_3O_4 NPs can be directly coupled with amine (NH_2) group via a Mannich reaction, offering a direct method for Fe_3O_4 functionalization. Here we choose to conjugate a cyclic RGD peptide, c(RGDyK), to the NP surface. This head-to-tail cyclized RGD peptide has been proven to target specifically to the activated endothelial cells and various tumor cells expressing cell adhesion molecule integrin $\alpha_v\beta_3$.[11] The coupling via a Mannich reaction was performed by mixing the MC-Fe_3O_4 NPs with c(RGDyK) and formaldehyde (HCHO) in DMF (Scheme 1).[8]

The RGD coated Fe_3O_4 NPs are stable for months without precipitation in aqueous dispersion (Figure S3). The overall size of the particles, measured by dynamic light scattering (DLS) in water, is 8.4 ± 1.0 nm (Supporting Information, Figure S3), close to the simple addition of the ∼4.5 nm core (Figure 1A) and ∼2 nm coating of 4-MC + c(RGDyK). The matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) shows various M/Z peaks (Figure S4) that confirm the successful conjugation of c(RGDyK) to 4-MC. The number of RGD peptide per particle was estimated to be 100−200.[8]

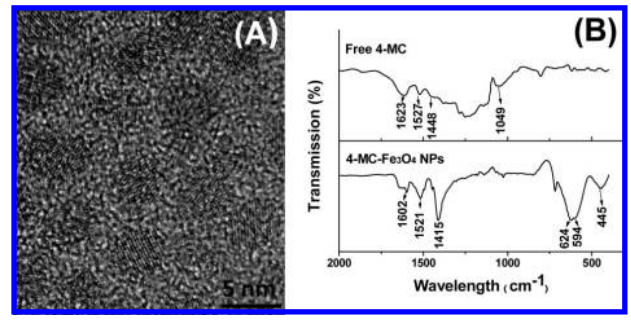


Figure 1. (A) HRTEM image of the as-synthesized, 4.5 nm MC-Fe_3O_4 NPs; (B) FTIR spectra of the free 4-MC and MC-Fe_3O_4 NPs.

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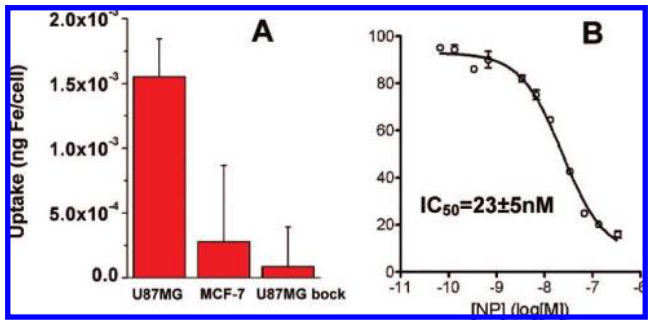

Figure 2. (A) Cell uptake of c(RGDyK)-MC-Fe_3O_4 NPs by U87MG, MCF-7, and U87MG + c(RGDyK) block, and (B) the c(RGDyK)-MC-Fe_3O_4 NP concentration dependent replacement of ^{125}I-echistatin on U87MG cells.

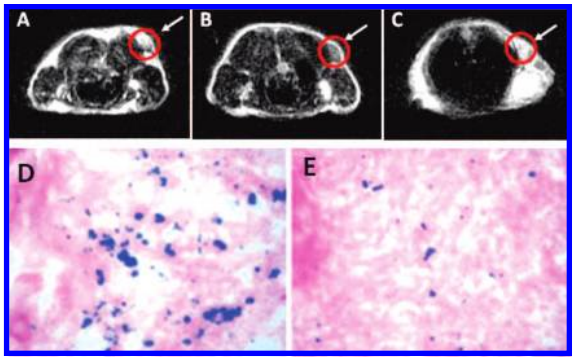

Figure 3. MRI of the cross section of the U87MG tumors implanted in mice: (A) without NPs, (B) with the injection of 300 μg of c(RGDyK)-MC-Fe_3O_4 NPs, and (C) with the injection of c(RGDyK)-MC-Fe_3O_4 NPs and blocking dose of c(RGDyK); and Prussian blue staining of U87MG tumors in the presence of (D) c(RGDyK)-MC-Fe_3O_4 NPs and (E) c(RGDyK)-MC-Fe_3O_4 NPs plus blocking dose of c(RGDyK).

To study integrin targeting specificity of the c(RGDyK)-MC-Fe_3O_4 NPs, we incubated these NPs with both U87MG human glioblastoma (high $\alpha_v\beta_3$) and MCF-7 human breast cancer (low $\alpha_v\beta_3$) cell lines at 37 °C for 30 min. The iron concentration within the cells were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES).[8] Figure 2A shows the Fe uptake for U87MG cells, which is about 5-fold higher that of MCF-7 cells. The accumulation of NPs in U87MG cells is effectively inhibited in the presence of the blocking dose (2 μM) of c(RGDyK), further demonstrating integrin specificity of c(RGDyK)-MC-Fe_3O_4 NPs. The c(RGDyK)-MC-Fe_3O_4 NPs also show good binding ability to integrin. This is confirmed by the displacement competitive binding assay using $\alpha_v\beta_3$ positive U87MG cells, as shown in Figure 2B. The c(RGDyK)-MC-Fe_3O_4 NPs inhibit ^{125}I-echistatin binding to integrin in a concentration dependent manner. The IC_{50} value, used to measure the affinity of the binding agents, is calculated to be 23 $\pm$ 5 nM for NPs, which is significantly higher than monomeric RGD peptide (250 $\pm$ 60 nM). The enhanced binding affinity is from the multivalent effect as discussed previously.[3,12]

The targeting ability of the c(RGDyK)-MC-Fe_3O_4 NPs to integrin $\alpha_v\beta_3$ in vivo was evaluated by T_2-weighted fast spin−echo MR imaging with mice bearing U87MG tumors. The r_2 relaxavity of the c(RGDyK)-MC-Fe_3O_4 NPs was measured to be 165 $mM^{-1}s^{-1}$ (Figure S5), which is larger than that of the commercial Feridex NPs (104 mM^{-1} s^{-1}) with similar core size.[13] This larger r_2 value is likely caused by the stronger field perturbation around the NPs due to the thin c(RGDyK) coating. The tumor MR signal intensity decreased significantly (42 $\pm$ 5%) after injection of c(RGDyK)-MC-Fe_3O_4 NPs (Figure 3A,B). Such signal reduction was inhibited (15 $\pm$ 3%) in the presence of a blocking dose of c(RGDyK) peptide (10 mg/kg) (Figure 3C), proving the specificity of the particle targeting in vivo. To further investigate the particle distribution in various organs, the animals were sacrificed immediately after the MR scan at 4 h time point, and Prussian blue staining was employed to visualize the disposition of the c(RGDyK)-MC-Fe_3O_4 NPs without and with the blocking dose of c(RGDyK) peptide (Figure 3D,E). Prominent blue spots on the U87MG tumor slices were observed (Figure 3D) and the blue spots were reduced in the presence of free c(RGDyK) (Figure 3E), further confirming that the accumulation of c(RGDyK)-MC-Fe_3O_4 NPs was mediated by integrin $\alpha_v\beta_3$ binding. Additionally, the c(RGDyK)-MC-Fe_3O_4 NPs were rarely seen in kidneys and muscle, indicating that the NPs sustained enough circulation time for targeting, even though some deposition in both liver and spleen were seen (Figure S6). The NPs in the tumor were mostly localized on the integrin expressing tumor vasculature and tumor cells with little to no macrophage uptake (Figure S7).

This report introduces a novel way of synthesizing and functionalizing ultrasmall Fe_3O_4 NPs as contrast agents for potential in vivo tumor detection using MRI. The Fe_3O_4 NPs are synthesized by thermal decomposition of $Fe(CO)_5$ in the presence of 4-MC followed by air oxidation. Mannich reaction is applied to couple NH_2-terminated peptide c(RGDyK), and the c(RGDyK)-MC-Fe_3O_4 NPs show the desired biocompatibility and specificity to U87MG tumor cells. Compared to the free RGD peptide, the c(RGDyk)-MC-Fe_3O_4 NPs with desired size could dramatically increase the cellular uptake due to the multivalent binding. This new NP functionalization strategy can be readily extended to couple other bioactive molecules with amine functional group to Fe_3O_4 NPs. Further work on extravasation ability of the biofunctionalized IONPs for target-specific delivery is underway.

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Supporting Information Available: Fe_3O_4 NP synthesis, surface functionalization and characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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